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Plant adaptations to severely phosphorus-impoverished soils

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Abstract

Mycorrhizas play a pivotal role in phosphorus (P) acquisition of plant roots, by enhancing the soil volume that can be explored. Non-mycorrhizal plant species typically occur either in relatively fertile soil or on soil with a very low P availability, where there is insufficient P in the soil solution for mycorrhizal hyphae to be effective. Soils with a very low P availability are either old and severely weathered or relatively young with high concentrations of oxides and hydroxides of aluminium and iron that sorb P. In such soils, cluster roots and other specialised roots that release P-mobilising carboxylates are more effective than mycorrhizas. Cluster roots are ephemeral structures that release carboxylates in an exudative burst. The carboxylates mobilise sparingly-available sources of soil P. The relative investment of biomass in cluster roots and the amount of carboxylates that are released during the exudative burst differ between species on severely weathered soils with a low total P concentration and species on young soils with high total P concentrations but low P availability. Taking a modelling approach, we explore how the optimal cluster-root strategy depends on soil characteristics, thus offering insights for plant breeders interested in developing crop plants with optimal cluster-root strategies.

Strategies of phosphorus acquisition in mycorrhizal and non-mycorrhizal species

Mycorrhizal structures, which arise from symbiotic associations between plants and fungi, occur in most terrestrial higher plants [1]. They play a pivotal role in acquisition of poorly-mobile nutrients, in particular phosphorus (P). Non-mycorrhizal species are relatively common in young landscapes on sites that contain the most exchangeable P, *e.g.*, in Swedish rocky habitats [2]. These are referred to as the 'Brassicaceae type' [3], after a well-known non-mycorrhizal family. Conversely, on the world's most P-impoverished soils such as the sandplains in south-western Australia [4], the Cape Floristic Region in South Africa [4], and the *campos rupestres* in Brazil [5], non-mycorrhizal species are also very common. In south-western Australia, their frequency increases with decreasing soil P concentration [6]. This group of non-mycorrhizal species is referred to as the 'Proteaceae type' [3], after a family common on severely P-impoverished soils in Australia and South Africa. In summary, non-mycorrhizal species occur at both ends of a soil fertility gradient. The Brassicaceae type is associated with fertile soils, whereas the Proteaceae type is found on severely P-impoverished soils [3].

To understand why non-mycorrhizal species are common on severely P-impoverished soils requires knowledge about P in soil. Only some of this P is in the soil solution; the rest is adsorbed onto or

absorbed by soil particles. Since adsorption and absorption are hard to separate, the process is usually referred to as 'sorption', a non-committal term coined by McBain [7]. In soil, goethite, named after the German poet and philosopher J.W. von Goethe, who had a keen interest in minerals, is an iron-bearing hydroxide mineral that tightly sorbs P. When phosphate is added to goethite, most of the added P is sorbed to the mineral, until a significant fraction of the sites that can bind P are occupied (Fig. 1) [8]. Adding more P then increases the concentration of P in solution. When *Lolium perenne* (ryegrass) is grown in pots with goethite, no P is readily available to ryegrass until >40% of the goethite surface is covered with phosphate ions. The P availability then increases till the concentration in solution reaches 2 μM, when 75% of all the P-binding sites of goethite are covered. Arbuscular mycorrhizal fungi increase the P availability for ryegrass at a concentration between 0.5 and 2 μM (60-70% coverage of the goethite surface). Neither below nor above that concentration range do arbuscular mycorrhizal fungi promote the growth of ryegrass. Above the range, the uninoculated ryegrass roots are just as effective as mycorrhizal roots at taking up P. Below that range, the mycorrhizal fungi are ineffective, but an alternative P-mining strategy based on displacing P from soil surfaces with citrate is more effective [8,9].

Cluster roots are common in Proteaceae, but functionally similar roots occur in other families

If arbuscular mycorrhizas are ineffective at very low soil P concentrations, is there evidence that plants that release citrate from roots can be more effective in soils that either contain very little total P or in which P is strongly sorbed? Indeed there is. Most Proteaceae are non-mycorrhizal, and in south-western Australia they predominantly occur on ancient soils that contain very little P [4]. These species produce proteoid or cluster roots (Fig. 2) [10]; *Hakea prostrata* (Proteaceae) releases carboxylates (organic anions) in an exudative burst [11]. In southern South America, Proteaceae occur on young acidic soils with very high total P concentrations, but with a low P availability, due to strong sorption of P to oxides and hydroxides of iron and aluminium [12]; *Embothrium coccineum* releases predominantly citrate [13]. In tropical rainforests in north-eastern Australia, a mycorrhizal species without cluster roots, *Placospermum coriaceum*, occurs, together with non-mycorrhizal species with cluster roots [14]. When grown at a very low soil P concentration, the non-mycorrhizal species with cluster roots grow better than the mycorrhizal species without cluster roots (Fig. 3), confirming that cluster roots are better than mycorrhizas at very low soil P availability.

Similar results are found with crop species. Comparing the response to applied soil P of a non-mycorrhizal crop species with cluster roots, *i.e. Lupinus albus* (white lupin), with that of a mycorrhizal species without cluster roots, *i.e. Triticum aestivum* (wheat), shows similar results to those in Fig. 3 [15]. The genus *Lupinus* is non-mycorrhizal, with only a small number known to produce cluster roots [16]. However, many that do not produce cluster roots do produce relatively large amounts of carboxylates, *e.g.*, *L. angustifolius* (narrow-leaf lupin) [17]. Narrow-leaf lupin produces more biomass at a low P supply than wheat does, but not as much as white lupin [16]. That is, the combination of programmed production and release of carboxylates and the structure that allows the local build-up of high concentrations of released carboxylates in the rhizosphere is ideally suited to access soil P when the P concentration in the soil solution is very low [18].

Cluster roots do not only occur in Proteaceae and some Fabaceae, but are also common in actinorhizal families [9,10]. Moreover, there are other structures that are morphologically very different, but functionally similar to cluster roots, *e.g.*, dauciform roots in many non-mycorrhizal

Cyperaceae [19,20], non-mycorrhizal sand-binding roots in *Discocactus placentiformis* (Cactaceae) [21], and capillaroid roots in *Lyginia barbata* (Anarthriaceae) (M.W. Shane, pers. comm.). Where investigated, leaves of these non-mycorrhizal carboxylate-releasing plants have high concentrations of manganese, because the carboxylates also mobilise this micronutrient [22]. Using leaf manganese concentrations as a proxy for whether a species uses carboxylates to mobilise P indicates that this strategy is quite common on soils where soil P availability is very low [5,23].

The role of phytohormones and sugars in cluster-root formation

Lateral root formation is regulated by a complex network involving phytohormones and nutrients. Auxin is the main actor in promoting lateral root production [24], initiating the development of lateral root primordia in the root pericycle that give rise to lateral roots. Cytokinins act on root founder cells to inhibit lateral root production [25]. Abscisic acid [26] and high ethylene concentrations [27] also negatively impact on lateral root production.

High P concentrations inhibit lateral root production, but enhance primary root elongation, while P starvation leads to the opposite phenotype. On the other hand, roots can sense specifically the presence of P and nitrogen (N) in the soil and respond by producing a large number of lateral roots in nutrient-rich patches [28]. For plants grown at a high P supply that do not normally form cluster roots, indoleacetic acid and its analogue naphthalene acetic acid can promote cluster-root formation [29,30]. In contrast, auxin-transport inhibitors suppress the formation of lateral roots as well as cluster roots [29]. Measurements of free auxin revealed a steep gradient within the root, with auxin concentrations highest in root apices and lowest in mature cluster roots [29]. Studies on cluster-root formation induced by external auxin showed that cluster-root production alone does not induce citrate efflux [31]. The importance of auxin in cluster-root formation has been highlighted in several recent transcriptomic studies, which also revealed the importance of performing detailed analyses with the different stages of cluster roots. A first study, comparing cluster roots, non-cluster roots and leaves in response to P supply, showed that several auxin-related genes are up-regulated in cluster roots at a low P supply [32]. Two subsequent studies comparing the different stages of cluster roots and the root apex showed a differential gene-expression pattern for the different stages of cluster roots [33,34]. Auxin-dependent transcription factors and genes involved in auxin biosynthesis and transport are most highly expressed before cluster-root emergence, and decline gradually during maturation [33,34]; genes are mainly expressed in the root apex and in younger stages of cluster roots. In one study [34], a gene implicated in brassinosteroid biosynthesis behaved similarly to many auxin-related genes. Brassinosteroids also participate in the regulation of lateral root formation [35].

Interestingly, sucrose is also involved in inducing cluster roots. Sugars act as signals for carbohydrate metabolism and control plant growth [36]. Under P starvation, more sucrose is allocated to the root system, allowing extensive growth and changing the shoot-root ratio for biomass in most plants [37]. However, sucrose is not only an energy source, but also a signalling compound (*e.g.*, [38,39]). Two reports have shown that in *Lupinus albus* cluster roots are formed in the presence of sufficient P supply when sucrose is supplied to the medium [34,40]. This is not due to an osmotic effect, since sorbitol at similar concentrations has no effect on cluster-root formation. Contrasting results have been presented for the effect of hexoses. Zhou et al. [40] observed both glucose and fructose inducing cluster-root formation, while Wang et al. [41] reported that the effect was specific for

sucrose. Different growth conditions were used in the two studies, and this may have had an impact. While Wang et al. [41] used a sterile hydroponic system, Zhou et al. [40] grew their plants on agar. Growth on agar inhibits the formation of a large number of cluster roots and may also have negative effects for nutrient and oxygen availability. The exudation activity of cluster roots was only partially established in sucrose-induced cluster roots. While acidification of the medium, although weaker than under low-P conditions, still took place for plants grown in the presence of sucrose and P, citrate exudation was not detectable. In line with this observation, the putative citrate exporter gene *L. albus MULTIDRUG AND TOXIN EFFLUX* (*LaMATE*), was not induced under these conditions [33,34]. Expression of genes encoding PEP carboxylase (*LaPEPC3*) and a high-affinity phosphate transporter (*LaPT1*) is much stronger in P-starved plants in the presence of sucrose than in its absence, indicating that there is a synergistic effect of P starvation and carbohydrate availability in cluster-root metabolism.

Recently, an additional player in cluster-root formation was discovered: nitric oxide. Wang et al. [42] showed that P deficiency induces nitric oxide production. As for auxin, the levels are highest in the pre-emergent stage, and decrease during cluster-root maturation. Under P-starvation conditions, addition of the nitric oxide donor sodium nitroprusside results in an increased number of cluster roots and lateral roots. Concomitantly, citrate exudation is enhanced. No formation of cluster roots occurs with addition of the donor under P-sufficient conditions, indicating that the nitric oxide signal has to be associated with a P-starvation signal to become active. Nitric oxide is also involved in cluster-root formation induced by iron (Fe) deficiency [43].

Cytokinins are important components in regulating lateral root formation, and strong antagonists of auxin-mediated cluster-root formation [30,32]. The presence of cytokinins in the growth medium strongly suppresses cluster-root formation [29]. The first evidence that transcripts encoding cytokinin oxidase (CKX), which is involved in cytokinin degradation, are up-regulated in P-deficient cluster roots was shown by O'Rourke et al. [32]. Later studies showed that genes related to cytokinin sensing, such as the receptors, are predominantly expressed at the pre-emergence and juvenile cluster-root stage, when cytokinin concentrations are higher [33,34]. In contrast, genes encoding enzymes involved in cytokinin degradation are strongly up-regulated in mature cluster roots [33,34].

Strigolactones have an inhibitory effect on lateral root formation, but enhance root-hair production [44]. However, exposing white lupin roots to 1 µM of the artificial strigolactone GR24 did not affect cluster-root production (D.J. Strack, G.R. Flematti, A. Scaffidi, S.M., Smith and H. Lambers, unpubl.). The same concentration reduces lateral root formation in *Arabidopsis thaliana* [44].

The role of ethylene is controversial. Results on white lupin indicate that it has no impact on cluster-root formation [29], while in *Casuarina glauca* inhibition of ethylene biosynthesis results in an inhibition of Fe-deficiency-induced cluster roots [45]. Ethylene may play a role in controlling the length of the cluster-root rootlets, since low ethylene concentrations inhibit lateral root initiation and elongation [46]. In line with this hypothesis, RNA-seq data revealed that expression of genes involved in ethylene biosynthesis is very low at the pre-emergent and juvenile stage, but strongly up-regulated at the mature stage [34].

The results reported so far indicate that cluster-root formation is similar to that of other lateral roots. However, the position of the lateral roots is different, and hence, the pattern of signalling compounds inducing lateral-root formation differs. Interestingly, a recent paper reports on a so-far

uncharacterised carotenoid derivative required for the periodic branching of *Arabidopsis* roots [47]. We therefore speculate that either the different periodic production of this carotenoid derivative or a different perception of this molecule comprise at least one of the components required for the production of cluster roots.

Cluster-root metabolism

Citrate is a carboxylate that effectively solubilises phosphate bound to oxides and hydroxides of Fe and Al. White lupin clusters exude mainly citrate and malate [48,49], similar to what has been found in Proteaceae [11]. Concomitant acidification is also observed in some cluster-root producing species, but not in all [50], and even in lupins, potassium, sodium and magnesium together are more important cations to accompany organic anion efflux than protons are [48]. Since the natural habitats of most cluster-rooted species are not calcareous soils, acidification is not an effective strategy to mobilise P. Rather, proton release serves to maintain charge balance, which can also be achieved with other cations, and to inhibit bacterial growth, to minimise microbial breakdown of released citrate and maximise its effect on P mobilisation [51]. In alkaline soils, acidification does contribute to mobilisation of P, but even then, carboxylates are more important than protons [52].

Carboxylate exudation from cluster roots occurs in response to either P or Fe starvation [53]. The metabolic response to P starvation is similar in cluster-root-producing and non-cluster-root producing species. However, the degree of expression of metabolic genes may differ, and the changes observed during cluster-root formation are cluster-root specific. Enzymes involved in malate and citrate metabolism, such as PEP carboxylase [54,55] and aconitase [30], show greater and lower activities, respectively, in cluster roots of white lupin. PEP carboxylase is activated by deubiquitination followed by phosphorylation [56]. Activities of enzymes of carbohydrate metabolism, such as sucrose synthase, are also increased during cluster-root development [57]. An even faster expression of genes encoding the alternative oxidase precedes the citrate and malate exudation peak [11]. The authors hypothesised that this enhanced expression allows an increased flux of electrons through the mitochondrial electron chain, thereby regulating the ATP/NADH balance [11,58]. Recent work using RNA-seq has increased our insight into genes differentially expresssed during cluster-root development. In mature cluster roots, many transcripts encoding enzymes involved in glycolysis, the pentose phosphate pathway, the glyoxylate cycle, and mitochondrial ATP synthesis show increased abundance [33,34]. In juvenile cluster roots, most of the genes involved in the TCA cycle show increased transcript levels, whereas they show lower abundance in mature cluster roots [30]. This is most striking for pyruvate dehydrogenase [34] and isocitrate dehydrogenase [59], whose increased transcript abundance favours the production of citrate. Although similar approaches have been taken in the work of Secco et al. [33] and Wang et al [34], a direct comparison cannot be made in all points, since the developmental stages differed.

Genes for cell-wall synthesis, which requires much energy, show lower transcript levels in mature cluster roots than in juvenile cluster roots, probably reflecting the fact that these roots stopped growing. In addition to genes involved in acclimation to a low P availability, genes involved in N and Fe metabolism are also induced in mature cluster roots. While the results obtained for N metabolism, including nucleotide degradation, appear to make sense, the fact that genes involved in Fe-starvation responses are up-regulated even in the presence of sufficient Fe is surprising. As in other plants grown under P-limiting conditions, the genes encoding enzymes involved in

biosynthesis of phenolics are also up-regulated in juvenile and mature cluster roots [34]. Concomitantly with the altered expression of genes encoding metabolic enzymes, the expression of genes encoding transporters is also induced [34]. Increased P-transport activity and expression of genes encoding P transporters have been reported [60]. Furthermore, a *L. albus* MATE homologue to the citrate exporter FRD3 (ferric reductase defective 3) in *Arabidopsis* is highly induced in cluster roots [61]. Plasma-membrane H^+-ATPases also show increased activity [62], and proton and citrate exudation are correlated, although protons are not the only counterions during carboxylate release [48]. The recent RNA-seq results confirmed earlier results and show that a large number of genes encoding other transporters are induced, including transporters for sugars, nitrate, amino acids, sulfate and potassium [34]. Interestingly, a gene encoding a putative malate channel of the aluminium-activated malate transporter (ALMT) family is also up-regulated when mature cluster roots are compared with juvenile ones [34]. This is somewhat surprising, since mature cluster roots of white lupin exude less malate than citrate. However, it should be taken into account that malate exudation in mature cluster roots is still substantial and that ALMTs are not only permeable for malate, but also for anions such as chloride and sulfate.

In conclusion, most of the effects in other plant roots experiencing P starvation are also observed in cluster roots. However, due to their high exudation capacity, the metabolic pathways allowing the production of large amounts of carboxylates are induced at an extremely high level and undergo a clearly-defined pattern from primordia to the mature stage of a cluster root.

Variation in structure and functioning of cluster roots as related to soil characteristics

With so many structures and species showing P-mobilising carboxylate releasing strategies, are there some that function better than others as dependent on soil conditions [13]? To address this question, we developed a simple theoretical model to predict how P uptake over time might vary with different rates of exudate release and in soils with varying levels of bound P. The model represents P uptake by the root, based on Michaelis-Menten kinetics; a constant rate of exudate production; diffusion of P and exudates through the soil volume; and displacement of bound P by exudates. Details of the model are provided in Supplementary Information. The model clearly predicts that P uptake is faster for a longer period in soils with a larger total P concentration compared with that in soils with less P, regardless of exudation rate (Fig. 4). Furthermore, reducing exudation rate has relatively little effect on P uptake over time in low-P soils, but in high-P soils reducing exudation rate has a much greater effect. This supports the assertion [13] that in high-P soils the most efficient strategy is to construct a relatively small number of cluster roots, maintain them for a relatively long time, and release exudates at relatively rapid rates, while in in low-P soils the most efficient strategy is to construct a relatively large number of cluster roots, maintain them for a relatively short time, and produce exudates at relatively slow rates.

Roots of crop plants that are less reliant on P-fertiliser inputs

When P is applied to agricultural soils as fertiliser, some reacts with the soil, leading to accumulation of P in sparingly-available forms [63]. The P-acquiring strategies we have discussed in this review increase plant access to sparingly-available sources. Mycorrhizal P-scavenging strategies are most suitable when P is limiting, but there is sufficient P in the soil solution out of reach of roots and root hairs. Carboxylate-releasing P-mining strategies, on the other hand, are more effective when there is very little P in the soil solution, and most P is sorbed onto soil particles.

Soils differ profoundly in the total amount of P they contain as well as in the proportion of soil P that is readily available. Sandy soils in south-western Australia, for example, contain little total P and plant-available P; a range of carboxylate-releasing P-mining strategies have evolved in different plant families in different parts of the world, including species that are used as crop plants for food or fibre production [64]. The specialised cluster-root structures in south-western Australia are short-lived and release relatively large amounts of carboxylates when compared with mycorrhizal species, but not as much as those in Proteaceae from southern South America which occur on young volcanic soils [13]. Such young volcanic soils contain much larger total amounts of P, but due to their low pH and strong P-sorption, the availability of soil P here is barely greater than that of the sandy soils in south-western Australia. Here, faster release of carboxylates for a slightly longer time leads to a greater return of P (Fig. 4). This information can be used to optimise the carboxylate-releasing strategy for crop plants for soils with different characteristics. This is possibly just as important in calcareous soils, if these contain large amounts of P, but with most of this being bound to calcium. However, calcareous sand dunes in south-western Australia are predominantly inhabited by arbuscular mycorrhizal species [23], possibly because the vegetation is primarily limited by N, rather than P [65]. This is possibly also the case for chalk grassland in Europe [66]. It has recently been proposed to use leaf manganese concentrations as a proxy for carboxylate-releasing strategies of studied plants, when screening for plants in ecology as well as for plant-breeding purposes [22].

When breeding for optimal strategies to acquire soil P, soil characteristics must be taken into account. On relatively fertile soils, where productivity is limited by P, mycorrhizal strategies are desirable. When a large fraction of total soil P is sorbed, carboxylate-releasing strategies are desirable. Some species use this strategy when non-mycorrhizal, suppressing it when inoculated by mycorrhizas [67], showing there is no sharp boundary between mycorrhizal and carboxylate-

releasing species. Cluster roots are likely the very best strategy when P is very poorly available. When the total amount of soil P is low, the optimal strategy is for plants to produce many cluster roots that are very short-lived and release carboxylates for a few days only. When the total amount of P is large, but the availability is low, the optimal strategy appears to be to produce less cluster roots that live somewhat longer, and release carboxylates at a faster rate for a slightly longer period. Since lupins are important crops on both soil types, we have the opportunity to breed lupins with the best strategy depending on where they will be grown. Likewise, other carboxylate-releasing strategies can likely be optimised for different soils.

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**This paper shows a tradeoff between carbon allocated to mycorrhizal symbionts and carbon used for carboxylate exudation. For a range of *Kennedia* species, it was shown that carboxylate release is less when the roots are colonised by arbuscular mycorrhizal fungi. The study illustrates that there is no sharp border between plants with a strategy to for mycorrhizal associations and plants that release carboxylates to acquire phosphorus. Rather, some plants appear to be able to shift between these strategies, depending on environmental conditions.

Figure legends

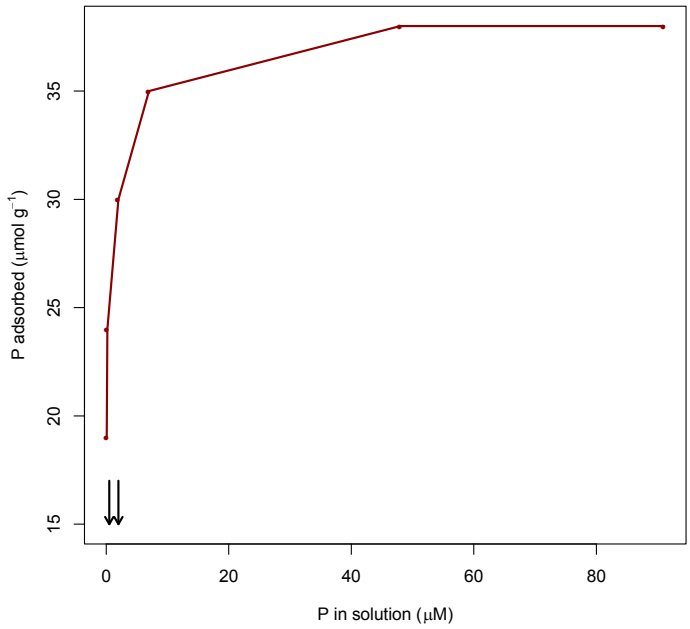

Figure 1. Phosphate-adsorption isotherm on goethite at pH 6.3 after two days shaking using $Ca(H_2PO_4)_2$, 0.01 M $CaCl_2$ at 20 ˚C. Following this, the phosphorus (P) concentration in the solution was measured as well as the amount of P sorbed to the goethite. The arrows denote the range over which inoculation of *Lolium perenne* (ryegrass) with an arbuscular mycorrhizal fungus enhances plant growth. Redrawn based on data in [8].

Figure 2. Cluster roots of *Hakea* species. Top: Active cluster roots of *Hakea ceratophylla*, photographed *in situ* in winter in Allison Baird reserve, Yule Brook. Bottom: Cluster roots of an unidentified *Hakea* species photographed after excavation close to the soil surface in Lesueur National Park, near Jurien Bay. Both locations are in south-western Australia. Photos: Hans Lambers.

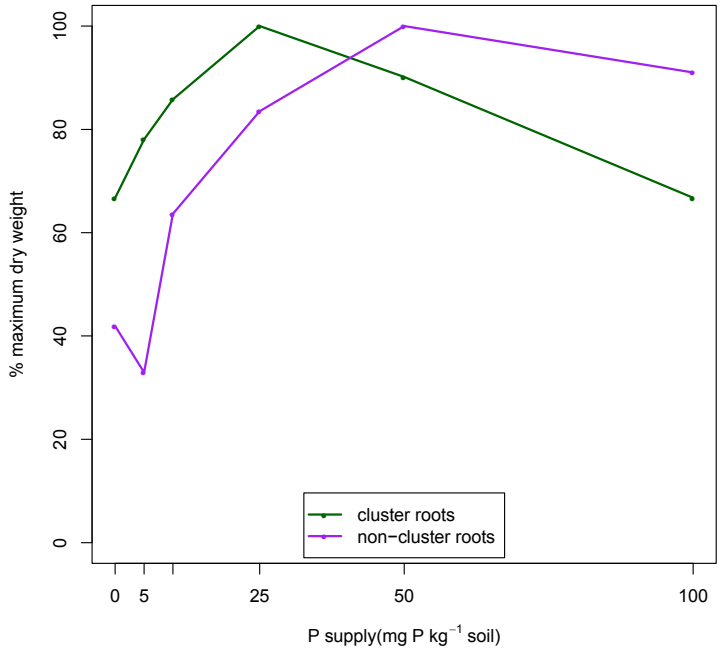

Figure 3. Comparative growth response (% of maximum shoot dry weight) to increasing phosphorus supply for seedlings of four species of rainforest Proteaceae from tropical rainforests in north-eastern Australia grown in a granitic soil. Only *Placospermum coriaceum* forms arbuscular mycorrhizas, but does not produce cluster roots. Combined data for three other species (*Darlingia darlingiana*, *Carnarvonia araliifolia* var. *montana* and *Musgravea heterophylla*) pertain to species that form cluster roots, but are non-mycorrhizal. Redrawn after [14].

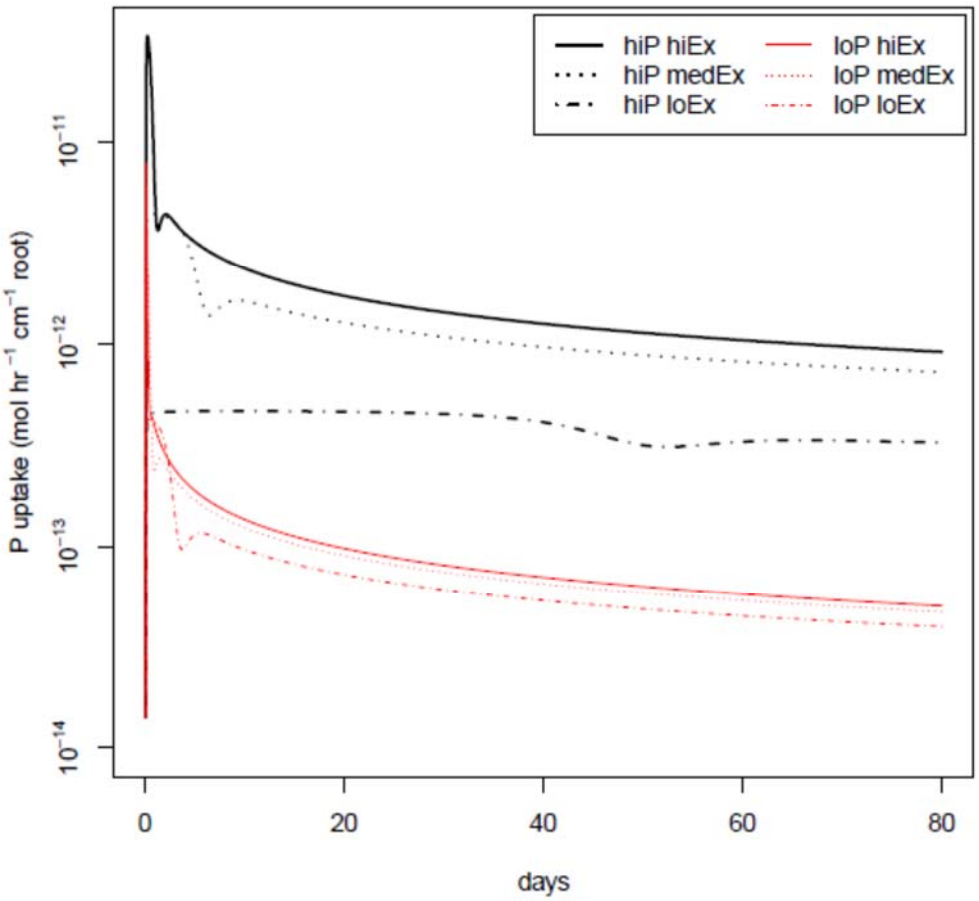

Figure 4. Estimated phosphorus (P) uptake over time by rootlets with different rates of carboxylate exudation (loEx, medEx, hiEx: $5x10^{-6}$, $5x10^{-7}$, $5x10^{-8}$ mol cm^{-2} root surface) in soils with different levels of available P (loP, hiP: 2.5, 50 mol m^{-3} soil).